DECLARATION

I, Hiroji KUMAZAWA, of 9-33-1-308 Kameido, Koto-ku, Tokyo, Japan, do solemnly declare that I, the translator of the documents attached, am well acquainted with the English and Japanese languages and certify that the following is a true translation to the best of my knowledge and belief.

Dated this 14th day of July, 1992.

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24.07.92

PATENT OFFICE

JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this office.

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SPECIFICATION

[Title of the Invention]

Process for the Expression of Human Protein Disulfide Isomerase Gene and Process for the Preparation of Polypeptides by Co-Expression with the Gene

[Claims]

1. A fusion gene for use in an expression of human protein disulfide isomerase, which is composed of a DNA fragment coding for a human serum albumin prepro sequence and a gene coding for said isomerase.

2. A fusion gene for use in an expression of human protein disulfide isomerase, the fusion gene being composed of a DNA fragment coding for a human serum albumin prepro sequence and a gene coding for said isomerase, characterized in that the fusion gene has a base sequence coding for the -24 to +491 amino acid sequence shown in SEQ ID NO:2.

3. A fusion gene according to claim 2, wherein said base sequence is the sequence between nucleotide 1 and nucleotide 1545 shown in SEQ ID NO:2.

4. A replicable expression vector capable of expressing the fusion gene according to any one of claims 1-3 in a host cell.

5. A transformant obtained by transforming a host cell with the expression vector according to claim 4.

6. A transformant according to claim 5, wherein the host cell is a yeast cell.

7. A process for the preparation of a recombinant human protein disulfide isomerase, characterized by expressing the fusion gene according to any one of claims 1-3, in the transformant according to claims 5 or 6.

8. A process according to claim 7, which comprises the following steps of:

constracting a replicable expression vector capable of expressing the fusion gene according to any one of claims 1-3 in a host cell;

transforming the host cell with said expression vector to obtain a transformant;

culturing said transformant under the conditions capable of expressing said fusion gene, to excrete a recombinant human protein disulfide isomerase; and

recovering said recombinant human protein disulfide isomerase.

9. A process according to claim 8, wherein the recombinant human disulfide isomerase excreted is separated and recovered by means of hydrophobic column chromatography.

10. A recombinant human protein disulfide isomerase having an amino acid sequence from position 1 to position 491 shown in SEQ ID NO: 3, the isomerase being obtained by the process according to any one of claims 7-9.

11. A transformant comprising, in a co-expressible state, the fusion gene according to any one of the claims 1-3 and a foreign gene coding for a polypeptide to be produced.

12. A transformant according to claim 11, which is a transformed yeast cell.

13. A transformant according to claim 11, wherein said foreign gene is a gene coding for human serum albumin.

14. A process for the preparation of a polypeptide, which comprises the following steps of:

co-expressing a human protein disulfide isomerase gene and a foreign gene coding for the polypeptide to be produced, in the transformant according to any one of claims 11-13 so as to produce the polypeptide; and

recovering said polypeptide.

15. A process according to claim 14, wherein said polypeptide is human serum albumin.

[Detailed Description of the Invention]

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(Addressed To)	Mr. Satoshi UEMATSU, Commissioner of Patent Office
(Title of Invention)	"PROCESS FOR THE EXPRESSION OF HUMAN PROTEIN DISULFIDE ISOMERASE GENE AND PROCESS FOR THE PREPARATION OF POLYPEPTIDES BY CO-EXPRESSION WITH THE GENE"
(Number of Claims)	15
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[Field of the Invention]

This invention relates to an expression of a gene coding for protein disulfide isomerase which is an enzyme for enhancing the formation of a high-order structure of polypeptide by catalyzing an exchange reaction of a disulfide bond(s) in the polypeptide. The present invention also relates to a co-expression of said gene with a foreign gene coding for a useful polypeptide.

[Prior Art]

Studies on the *in vitro* refolding of denatured proteins have revealed the presence of both isomerization reactions of a disulfide bond and of a proline peptide as factors for determining a folding rate of polypeptides (Freedman, *Cell*, vol.57, pp.1069 - 1072, 1989; Fisher and Schmid, *Biochemistry*, vol.29, pp.2205 - 2212, 1990). As enzymes which catalyze these slow reactions during the polypeptide folding, peptidyl prolyl cis-trans isomerase (PPI) has been found in the latter case, and protein disulfide isomerase (PDI) and thioredoxin in the former case. According to *in vitro* experiments, these enzymes rise a refolding rate of denatured proteins, thus indicating a possibility of applying them to the *in vitro* refolding of inactive proteins produced by genetic engineering techniques (Schein, *Bio/Technology*, vol.7, pp.1141 - 1148, 1989; J. Udaka, *Nippon Nogei Kagaku Kaishi*, vol.64, pp.1035 - 1038, 1990).

Since PDI is soluble in water and can be isolated relatively easily from the liver of mammals, its properties as a catalyst have been studied in detail. PDI catalyzes the exchange reaction between thiol/disulfide bonds and is capable of undergoing formation, isomerization or reduction of the disulfide bond in protein substrates (Freedman, *Cell*, vol.57, pp.1069 - 1072, 1989). It is known that, *in vitro*, PDI enhances the formation or exchange reaction of a disulfide linkage(s) in molecules of a single domain protein such as RNase and of a multiple domain protein such as serum albumin, or enhances the formation of an intermolecular disulfide bond(s) in a protein having a subunit structure such as immunoglobulin, procollagen or the like (Freedman, *Nature*, vol.329, p.196, 1987).

PDI from Mammals exists usually as a homodimer of the polypeptide having a molecular weight of about 57,000 and shows a highly acidic pI value (4.2 to 4.3).

The PDI gene from rat liver has been isolated. The amino acid sequence deduced from the DNA sequence of the PDI gene indicated that PDI has an intramolecular duplicate structure consisting of two homologous units. One of these two homologous units has a homology to the amino acid sequence of thioredoxin, indicating that its active site has an amino acid sequence similar to that of thioredoxin (Edman *et al.*, *Nature*, vol.317, pp.267 - 270, 1985). Thioredoxin enhances the reduction of a disulfide bond in insulin and the exchange reaction of a disulfide bond in RNase *in vivo*, which indicate that thioredoxin plays a similar role to PDI in the *in vivo* folding process of proteins (Pigiet and Schuster, *Proc. Natl. Acad. Sci.*, USA, vol.83, pp.7643 - 7647, 1986).

Although the amount of PDI present in a living body differs depending on the type of tissues and the differentiation stage of cells, such a difference is correlated with the existence of certain

secretory proteins. In addition, PDI is localized abundantly in the endoplasmic reticulum through which a protein is known to pass during its secretion. On the basis of these facts, it is assumed that PDI concerns with the formation of a disulfide bond(s) in secretory proteins newly synthesized within cells. Such an assumption is supported by the results of a study on the biosynthesis of γ-gliadin in a cell-free protein synthesis system, that the formation of a disulfide bond in conjunction with the translation of γ-gliadin hardly occurs when an endoplasmic reticulum fraction from which PDI was washed out in advance is used, while the disulfide bond formation is restored by the addition of PDI (Bulleid and Freedman, Nature, vol.335, pp.649 - 651, 1988).

In addition to the disulfide bond formation, PDI concerns with other post-translational modifications of proteins. For example, the polyfunctional property of PDI in connection with the protein modifications has been suggested on the basis of its homology to a catalytic unit, β-subunit, of prolyl-4-hydroxylase which catalyzes hydroxylation of proline residues in collagen, to a glycosylation site binding protein that recognizes a signal sequence Asn-X-Ser/Thr of a peptide to which a sugar chain is bound during N-glycosylation process of synthetic protein (Pihlajaniemi *et al.*, *EMBO J.*, vol.6, pp.643 - 649, 1987; Geetha-Habib *et al.*, *Cell*, vol.54, pp.1053 - 1060, 1988), to a thyroid hormone binding protein (triiodo-L-thyronine binding protein; Cheng *et al.*, *J. Biol. Chem.*, vol.262, pp.11221 - 11227, 1987), etc. In addition to these facts, some molecular species having partly homologous amino acid sequences to PDI have been found though different from the PDI. For example,

certain gonadotropic hormones such as follitropin and lutropin contain amino acid sequences homologous to an amino acid sequence which is regarded as an active site of PDI, and these hormones catalyze the isomerization of a disulfide bond (Boniface *et al.*, *Science*, vol.247, pp.61 - 64, 1990). Also, phospholipase C, an enzyme which hydrolyzes phosphatidylinositol-4,5-bisphosphate into 1,2-diacyl glycerol and inositol-1,4,5-triphosphate, has a domain homologous to PDI in its molecule (Bennett *et al.*, *Nature*, vol.334, pp.268 - 270, 1988). In consequence, PDI and PDI-like molecules seem to concern in a markedly wide range of vital phenomena, both intracellularly and extracellularly.

Although the PDI has extensive functions as described above, a main effect of PDI is to form a protein (or a protein aggregate) having a natural higher-order structure by catalyzing the isomerization of an intramolecular or intermolecular disulfide bond(s). In many cases, however, an almost stoichiometric amount of PDI is required to attain an optimum reaction rate. It is expected therefore that an intramolecular or intermolecular isomerization rate of a disulfide bond will be slow when a disulfide isomerase has a low activity, and such a slow reaction rate will entail a low formation efficiency of a protein having a suitable disulfide bond(s). It is thought that such a low disulfide isomerase activity is one of the cause of the formation of insoluble molecular aggregates of various eukaryote-originated proteins (especially secretory proteins) in *Escherichia coli*. Although *E. coli* contains thioredoxin which is superior to PDI in terms of the activity as a disulfide reductase, the isomerase activity of thioredoxin is low.

On the contrary, since an intramolecular disulfide bond(s) can often be found in secretory proteins, it is thought that a disulfide bond activity resulted from disulfide isomerization is high in cells or tissues which have a high secretion ability. This was indicated strongly by a comparative study on the relative PDI mRNA contents in various rat tissues, in which the contents in organs were found to be liver > pancreas, kidney > lung > spermary, spleen > heart > brain in order (Edman *et al.*, *Nature*, vol.314, pp.267 - 270, 1985).

When an environment in a reduced state is given to a protein synthesis system, the formation of a disulfide bond which is necessary for the suitable folding of a polypeptide will be inhibited. Such an environment is generated for example in prokaryotic cells which have no compartments. Taking this into consideration, prokaryotic cells and eukaryotic cells may be different from each other in terms of factors concerning the formation of a disulfide bond and of an environment which enables its formation. When useful proteins (most of them are secretory proteins) is produced by recombinant DNA techniques, it is necessary to form a disulfide bond under certain conditions which are suitable for each protein to be produced. For this purpose, a suitable environment (compartment) should be formed in a host cell and a large amount of a disulfide-forming enzyme (i.e., disulfide isomerase) should be present which has a high affinity for the environment (compartment).

The above two points must be considered greatly when a protein having a disulfide bond(s) is produced effectively using recombinant DNA techniques.

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However, nothing is in practice known about an *in vivo* system in which PDI is contained in a large quantity in a suitable compartment where a useful target protein co-exsists, the PDI being capable of acting on the protein.

[Problems to be solved by the Invention]

In spite of the applicability of PDI to the *in vitro* refolding of denatured proteins and to the improved productivity of secretory proteins in cells, this enzyme has been prepared only by direct purification from the internal organs. In addition, there are no reports on the interspecific expression of PDI, and on the establishment of a process for its production by means of genetic engineering or a process in which the productivity of a useful polypeptide is improved by the combination of the PDI gene with a gene coding for the polypeptide.

It is objects of the present invention is to provide a fusion gene for use in the expression of PDI, which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and a gene coding for the PDI; an expression vector capable of expessing said fusion gene in a host cell; a transformant obtained by transforming a host cell with said vector; a process for the preparation of a recombinant human PDI by expessing the fusion gene in said transformant; and the recombinant PDI.

Other objects of the present invention are to provide a transformant containing, in a co-expressible state, both the above mentioned fusion gene and a foreign gene coding for a polypeptide to be produced; and a process for the production of the polypeptide by

co-expressing the human PDI gene and the foreign gene in said transformant so as to produce the polypeptide.

[Means to solve the Problems]

The present invention has been completed by finding an expression vector integrated a fusion gene for expression of PDI which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and a gene coding for human PDI.

The following describes the present invention in detail:

Clones containing a human PDI cDNA are isolated from the human liver and placenta λgt11 cDNA libraries (Clontech, US) by the following procedures:

An *E. coli* strain is infected with phage from the human liver and placenta λgt11 cDNA libraries, after which DNAs from the phage grown are fixed on a filter. Separately from this, positive clones are screened by hybridization using a 40 mer synthetic oligomer DNA as a probe which corresponds to the complementary strand of a nucleic acid sequence (243-282) of human proline 4-hydroxylase (the same protein as PDI) cDNA (Pihlajaniemi, T. *et al.*, *EMBO J.*, vol.6, p.643, 1987). The phage DNA obtained is digested with *Eco*RI, and the resultant 150 bp insert DNA is used as a probe for screening the PDI cDNA. Using the probe, the phage DNAs fixed on the filter are screened to isolate positive clones.

Thereafter, a plurality of positive clones obtained in such a manner are digested with *Eco*RI so as to isolate *Eco*RI insert DNA fragments, and a restriction map of the insert of each clone is made. From the comparison of these maps with that reported by

Pihlajaniemi *et al.*, it was estimated that the full length human PDI cDNA was covered by a clone (pHPDI16) from liver and a clone (pHPDIp4) from placenta.

Determination of DNA sequences of the two clones revealed that these clones encoded human PDI cDNA consisting of 2454 base pairs in full length as shown in the SEQ ID No:1. An amino acid sequence deduced from the DNA sequence is also shown in the SEQ ID No:1. In the amino acid sequence, a mature protein seems to be composed of 491 amino acids from Asp^{1} to Leu^{491}, and the 17 amino acid polypeptide preceding Asp^{1} seems to be a signal peptide.

According to the present invention, there is provided a fusion gene for use in the expression and production of PDI, which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and the aforementioned human PDI gene.

As shown in Fig. 1C, the fusion gene is constructed in general by arranging the preprosequence-encoding DNA fragment at the upstream side of the PDI gene. In this instance, however, a leader sequence for transporting human PDI into an appropriate compartment (considered to be endoplasmic reticulum) is not always limited to the HSA prepro-sequence, and other signal sequences or prepro-sequences may also be used as the leader sequence.

More particularly, said fusion gene may be prepared as follows:

The aforementioned clones pHPDI16 and pHPDIp4 DNAs are double-digested with *Eco*RI/*Pst*I and *Pst*I/*Bam*HI, respectively, to produce DNA fragments of about 490 bp and about 1.3 kbp respectively, the fragments recovered are ligated with a plasmid vector pUC119 which was digested with *Eco*RI and *Bam*HI to produce phPDIEB in which a *Nae*I

cleavage site is then introduced into the boundary between the PDI signal sequence and the PDI sequence on the cDNA by the Kunkel's method (Kunkel, T.A., *Proc. Natl. Acad. Sci.*, USA, vol.82, p.488, 1985) so as to prepare phPDINae, and thereafter the phPDINae is digested with *NaeI* and *HindIII* to give a PDI DNA fragment of about 1.7 kb which does not contain the PDI signal sequence.

Separately from this, pUC119 is digested with *EcoRI*, and the resultant digest is ligated with the following *XhoI* linker:

5'-AATTCTCGAG

GAGCTCTTAA-5'.

After double-digesting the product with *XhoI* and *BamHI*, the digest is ligated with a prepro-sequence of HSA to produce pUC119Sig which is subsequently digested with *StuI* and *HindIII* to give a DNA fragment of about 3.2 kb (A method for synthesizing the HSA prepro-sequence will be described later in Examples).

Thereafter, the 1.7 kb DNA fragment from phPDINae and the 3.2 kb DNA fragment from pUC119Sig origin are ligated together to produce phPDILy1 which is in turn digested with *EcoRI*, blunt-ended with Klenow fragment, and digested with *BamHI*, thereby giving a fusion gene in which a leader sequence is modified and in which the human PDI gene is fused to the downstream side of the HSA prepro-sequence (Fig. 2).

Both a process for the preparation of the fusion gene according to the present invention and an arrangement of its constituted genes are not limited to the above-described techniques, provided that said fusion gene has an ability for expressing PDI. Although analogs of the inventive fusion gene are not included within the

scope of the present invention, it is obvious that they can be prepared easily from a corresponding gene of any animal origin other than human.

According to one embodiment of the present invention, said fusion gene has a DNA sequence coding for the -24 to +491 amino acid sequence shown in the SEQ ID NO: 2. Instead of the PDI gene therein, a DNA sequence coding for the +1 to +491 amino acid sequence (Asp^{1}--Leu^{491}) shown in SEQ ID NO:1 may also be applied. In these instances, all genes which substantially have the same function as that of said DNA sequences, for example, derivatives having nucleotide sequences based on the degeneracy of codon, are included within the scope of the present invention. According to another embodiment of the present invention, an example of such a fusion gene includes the whole of the base sequence shown in SEQ ID NO: 2.

The present invention also provides a replicable expression vector capable of expressing the fusion gene of the present invention in a host cell.

An expression vector used for the insertion of the linked gene of the present invention thereinto should replicate in a host cell and have the ability for expressing therein. In general, such a useful vector contains replicon and regulatory sequences which are derived from a species compatible with a host to be used, as well as a replication origin and a marker sequence which enables selection of a phenotype from transformed cells.

As a vector for use in the construction of the expression vector, the plasmid pJDB-ADH-HSA-A (Fig. 1C) which has been

disclosed in Japanese Patent Application Laying-Open (KOKAI) No. 2-217384 filed by the present applicant may be used conveniently. This plasmid contains HSA cDNA, as well as yeast alcohol dehydrogenase I (ADH I) promoter, ADH I terminator, ampicillin resistance gene (Amp^R) and Leu2 gene. The HSA cDNA is removed from this plasmid by digesting it with *Xho*I, blunting with Klenow fragment, and then digesting with *Bam*HI. The 3'-end of the DNA fragment of about 8 kb thus obtained is dephosphorylated, and the resultant fragment is ligated with the aforementioned fusion gene of the present invention to give the expression plasmid pAHhPDILyl. In this process, other types of vectors can be used provided that they are capable of expressing the fusion gene.

The present invention further provides a transformant obtained by transforming a host cell with the expression vector of the invention.

Examples of such a host include prokaryotes such as *E. coli*, *Bacillus subtilis*, etc, and yeast. Preferred is a host cell capable of secreting the mature PDI via processing. Preferably, the host cell is yeast such as *Saccharomyces cerevisiae*, and, in particular, a yeast strain AH22 is suitably used in the case of a preparation of the transformant according to the present invention. It is obvious that eukaryotes other than yeast, for example animal cells, can be used as the host cell, although they are not included within the scope of the invention.. Incorporation of the expression vector into a host cell can be carried out easily by conventional means such as calcium chloride, protoplast(or spheroplast)-polyethylene glycol, electroporation, etc. When the plasmid pAHhPDILyl is used as an expression vector, a desired transformant may be obtained by culturing transformed cells on SD(-Leu) plate and screening colonies grown on the plate.

Accordingly, the present invention also provides a process for the preparation of a recombinant human PDI by expressing the fusion gene of the invention in a transformant prepared in the same manner as described above. In one enbodiment of the present invention, the process of the invention comprises the following steps of:

constructing an expression vector which can replicate in a host cell and express the fusion gene of the present invention therein;

isolating a host cell transformed with said expression vector;

culturing the obtained transformant under such conditions that the fusion gene can be expressed, thereby secreting said recombinant human PDI; and

recovering the recombinant PDI.

If the host is yeast, a human PDI precursor protein is processed to excrete the human recombinant PDI as a gene product. If microorganisms other than yeast, for example *E. coli* and *Bacillus subtilis*, are used as host, a non-processed human PDI precursor protein will be obtained.

The recombinant human PDI can be purified easily by separating the transformed cells from a cultured medium by centrifugation, disrupting the cells if necessary, concentrating the supernatant by ultrafiltration or the like, and then subjecting the concentrate to hydrophobic column chromatography. Though not particularly limited, TSK-gel Phenyl-5PW hydrophobic column (Tosoh, Japan) may be used in the chromatography. In this case, the recombinant human PDI may be

eluted by linear gradient of from 0.85 to 0 M ammonium sulfate in borate buffer (pH 8.0) containing KCl (Fig. 4). It was confirmed that the purified recombinant human PDI has a molecular weight of about 55 kDa based on the SDS-polyacrylamide gel electrophoresis analysis (Fig. 5), and practically has PDI activity as the results of determination of a degree of the refolding of scrambled ribonuclease A (see Examples).

In comparison with the natural type human PDI, it has been found that the recombinant human PDI thus prepared has the same amino acid sequence except that its N-terminal amino acid is changed from Asp to Gly. Accordingly, the present invention provides a recombinant human PDI having the amino acid sequence Gly^{1}---Leu^{491} consisting of 491 amino acids shown in SEQ ID NO:3.

The present invention further provides a transformant comprising a fusion gene which is composed of a human PDI gene and a DNA fragment coding for a HSA prepro-sequence, and a foreign gene coding for a polypeptide to be produced, in a co-expressible state.

The fusion gene and the foreign gene in the transformant may be located on the same or different chromosome(s), provided that they are mutually present in a co-expressible state. Transformation of a host cell can be carried out for example by inserting the fusion gene and the foreign gene into the same or different vector(s) and introducing the resulting vector or vectors into the host cell by conventional means such as calcium chloride, protoplast(or spheroplast)-polyethylene glycol, electroporation, etc.

The foreign gene may encode a polypeptide of any type, provided that the polypeptide contains a disulfide linkage(s) because the

catalytic effect of an amplified and expressed PDI, that is, acceleration of the formation or exchange reaction of a disulfide bond(s) in polypeptide, is directly exhibited. In addition, the present invention can be applied to a case in which the PDI activity exerts influence on proteins relating to gene expression, polypeptide folding or transport, thereby indirectly improving the productivity of PDI. According to the embodiment of the present invention, the foreign gene is a gene coding for human serum albumin (HSA).

The term "polypeptide" as used herein means a short- or long-chain peptide and a protein.

Examples of hosts include prokaryotes such as *E. coli*, *Bacillus subtilis*, etc and eukaryotes such as yeast, animal cells, etc. Preferred is a host cell capable of secreting a mature polypeptide through post-translational modification and processing, more preferably eukaryotes, and most preferably yeast.

The present invention also provides a process for producing a polypeptide, which comprises the following steps of:

co-expressing a human PDI gene and a foreign gene coding for the polypeptide to be produced, in the above-described transformant so as to produce the polypeptide; and

recovering the polypeptide.

When HSA and PDI were co-expressed in an HSA-producing yeast strain transformed with a human PDI expression plasmid in an appropriate medium according to the embodiment of the present invention, a secretion level of HSA was practically increased up to

about 60% in average in comparison with the case of a non-transformed HSA-producing yeast strain (pAH/HIS23) (Fig. 8).

Although we do not intend the present invention to restrict by theory, the increase in the secretion level of HSA by co-expression can be explained as follows:

HSA is a protein containing 17 disulfide bonds. It is known also that formation of its higher-order structure is enhanced in the presence of a stoichiometric amount of PDI in *in vitro* refolding experiments of a denatured protein.

HSA is secreted from the yeast strain HIS23 as a water-soluble molecule, but some of the HSA molecules are also detectable within the yeast cell. When the intracellular HSA was analyzed by SDS-polyacrylamide gel electrophoresis, it was ditected as a single band with the same mobility as that of a normal HSA molecule in the presence of a reducing agent, while detected as discontinuous bands having a larger molecular weight than normal HSA in the absence of a reducing agent, clearly showing a different behavior from that of normal HSA. These results indicate that the presence of intracellular HSA molecules is based upon the incomplete formation of an intramolecular disulfide bond(s). In a yeast strain allowed PDI to co-express together with HSA, however, an intracellular HSA molecule was detected as a more narrow band on a SDS-polyacrylamide gel electrophoresed without a reducing agent when compared with an intracellular HSA sample prepared from a yeast strain which can not co-express a foreign PDI cDNA together with HSA. This indicate that PDI enhances the formation of a normal disulfide bond(s) in the HSA molecule and thereby assists the formation of the higher-order

structure of HSA molecule more efficiently. Accordingly, it is suggested that the co-expression of PDI reduces chances of causing the association of HSA molecules having unstable structure and their decomposition by proteases in the host cell, thereby increasing the secretion of HSA molecules.

When the amount of HSA mRNA in the HIS23 in which PDI was co-expressed is compared with the amount in a control without co-expression by means of northern blotting, increase in the amount of HSA mRNA can be found in the former cells in which the PDI gene was expressed. These results suggest that PDI exerts influence not only directly on HSA molecules but also on the transcription level of the HSA gene. However, it seems to be reasonable that the increment of HSA production level is based on the direct influence of PDI on HSA molecules by their coexistence in endoplasmic reticulum, because the increase in the amount of secreted HSA has a correlation to an increased level of the secretion of human PDI out of the yeast cell based upon the fusion of the PDI gene with the HSA prepro-sequence which plays a role in the intracellular transport into endoplasmic reticulum through a transmembrane process. In addition, when the amounts of HSA and PDI secreted from the HIS23 cells are compared with each other, PDI is secreted in several times larger amounts than HSA, and the level of human PDI detectable within the cells is also higher than HSA. These results, therefore, indicate that PDI is localized in the endoplasmic reticulum of the yeast cells in an enough amount to enhance the *in vitro* refolding of a denatured HSA, which also supports the direct effect of PDI on HSA.

Thus, it is highly possible that the effect of the co-expression of PDI on an increment of the amount of secreted HSA is based on the direct influence of PDI on the formation of the higher-order structure of HSA. In consequence, such a similar secretion-improving effect can also be expected in other general secretory proteins in which the formation of a disulfide bond(s) contributes to the formation and stability of their higher-order structures, by highly amplified co-expression of PDI in the same host cell.

The following non-limited examples will be provided to further illustrate the present invention.

[Examples]

Cloning of human PDI (protein disulfide isomerase) cDNA

About 100,000 clones of human liver λgt11 cDNA library (Clontech) were mixed with 500 µl of a culture of *E. coli* strain Y1090 which has been precultured overnight at 37°C in LB medium (1% Bacto-trypton, 1% NaCl and 0.5% yeast extract) supplemented with 0.2% maltose. After further adding 5 µl of 1 M $MgCl_2$ solution thereto, the mixture was incubated at 37°C for 10 minutes to infect the *E. coli* cells with the phage particles. The resulting cells were added to 50 ml of an LB top agar medium (LB medium, 10 mM $MgCl_2$ and 0.7% agarose), and then mixed and inoculated on a LB agar plate (23 cm x 23 cm). After solidifying the top agar medium, the plate was incubated overnight at 37°C so as to grow the phage particles. The phage particles obtained were transferred onto a filter (Hybond-N, Amersham). With the phage-attached side upward, the filter was

put for 1 minute on a 3MM filter paper (Whatman) which has been soaked in an alkaline solution (0.5 N NaOH and 0.15 M NaCl), and then for further 1 minute on the same filter paper which has been soaked in a neutral solution (1 M Tris-HCl (pH 7.5) and 1.5 M NaCl). Thereafter, the filter was washed with 2 x SSC solution (20 x SSC = 3 M NaCl + 0.3 M trisodium citrate), air-dried and then exposed to UV ray for 2 minutes so as to fix the phage DNA on the filter. Using the filter thus obtained, a screening of human PDI cDNA was carried out according to the following procedure:

As a probe to be used, a 40 mer oligomer DNA (5'-TGGCGTCCACCTTGGCCAACCTGATCTCGGAACCTTCTGC-3') which corresponds to the complementary chain of the 243-282 base sequence of human proline-4-hydroxylase (the same protein as PDI) cDNA (Pihlajaniemi, T. et *al.*, *EMBO J.*, vol.6, p.643, 1987) was synthesized using an automatic DNA synthesizer (Model 380B, Applied Biosystems).

The 5'-end of the synthesized DNA was labeled by phosphorylation, by incubating 20 pmoles of the DNA at 37°C for 60 minutes in 50 µl of 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 100 µCi [γ-^{32}P] ATP (~ 3000 Ci/mmol, Amersham) and 12 units of T4 polynucleotide kinase (Takara Shuzo, Japan). The filter obtained above was soaked at 37°C for 1 hour in the prehybridization solution which consists of 5 x Denhardt solution (100 x Denhardt solution = 2% bovine serum albumin + 2% Ficol 400 + 2% polyvinyl pyrrolidone), 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0), 0.1% sodium dodecyl sarcosinate and 20 µg/ml of an ultrasonic-treated salmon sperm DNA. The filter was further soaked in a hybridization solution (prepared by

supplementing the prehybridization solution with about 10^6 cpm/ml of the aforementioned labeled DNA) for 15 hours at 37°C. The resulting filter was washed with 2 x SSC solution at room temperature and then with 2 x SSC + 0.1% sodium dodecyl sarcosinate solution at 42°C for 30 minutes, followed by its exposure to an X-ray film (XAR-5, Kodak) overnight at -80°C. After development of the film, 8 positive signals were detected by the primary screening. Phage particles corresponding to those signals were recovered from the aforesaid plate by cutting it out as gel sections, soaked each of the gel sections in 1 ml of SM buffer (100 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and 0.01% gelatin), and left overnight at 4°C so as to recover the phage from the gel into the solution. When the 8 positive phages from the primary screening were further subjected to a second screening under the same conditions as those of the primary screening, only one of them remained as a positive clone. This clone was further subjected to a third screening in order to isolate it as a homogeneous positive clone.

A phage DNA was prepared from the positive clone obtained finally by the method of Leder et al. (Leder, P., Tiemeir, D. and Enquist, L., *Science*, vol.196, p.175, 1977). The thus prepared phage DNA (1/5 vol) was digested at 37°C for 1 hour in 50 µl of the digestion solution consisting of 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$, 6 mM mercaptoethanol, 0.1% gelatin, 20 µg/ml of ribonuclease A and 20 units of *EcoRI* (Nippon Gene, Japan). By 0.8% agarose gel electrophoresis of the resulting digest, it was found that this positive clone contains an insert DNA fragment of about 150 bp. The insert DNA was separated and purified using glass

powder (Gene Clean™, Bio-101). About 20 ng of the recovered DNA fragment and about 100 ng of pUC19 vector which has been digested with *EcoRI* were added to the mixture of Liquid A 20 µl and Liquid B 4 µl from the DNA ligation kit (Takara Shuzo, Japan), and the resulting mixture was then incubated at 16°C for 15 hours to obtain a recombinant plasmid in which both DNA fragments were linked together. Using 10 µl of this reaction mixture, transformation of *E. coli* strain TG1 was carried out by the Mandel's method (Mandel, M. and Higa, A., *J. Mol. Biol.*, vol.53, p.154, 1970). The transformant thus obtained was cultured overnight at 37°C in 100 ml of LB medium supplemented with 25 µg/ml of ampicillin, and a plasmid DNA was purified from the cultured cells by alkaline lysis method (Birnboim, H.C. and Doly, J., *Nucleic Acids Res.*, vol.7, p.1513, 1979). 10 µg of the plasmid DNA was digested at 37°C for 1 hour in 200 µl of the digestion solution consisting of 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$, 6 mM mercaptoethanol, 0.1% gelatin and 100 units of *EcoRI* (Nippon Gene, Japan). The digest was extracted with phenol, concentrated by ethanol precipitation and then subjected to 0.8% agarose gel electrophoresis. Thereafter, an insert DNA fragment of about 150 bp was recovered by glass powder technique, for use as a probe in the following PDI cDNA screening.

In order to obtain a clone which contains the full length human PDI cDNA, screenings were carried out again from about 50,000 clones of human liver λgt11 cDNA library and about 50,000 clones of human placenta λgt11 cDNA library (Clontech). Filters on which phage DNA molecules of the two libraries were fixed were prepared in the same manner as described in the foregoing. In this instance, about 100

ng of the aforementioned 150 bp human PDI cDNA fragment was isotope-labeled using [α-^{32}P] dCTP (>400 Ci/mmol, Amersham) and a nick translation kit (Amersham), and the labeled cDNA fragment was used as a probe in the screening. After soaking the above two filters in the aforementioned prehybridization solution for 1 hour at 60°C, the filters were further soaked in a hybridization solution (prepared by supplementing the prehybridization solution with about 10^6 cpm/ml of the labeled DNA) for 15 hours at 60°C. The resulting filters were washed with 2 x SSC solution at room temperature and then with 0.5 x SSC + 0.1% sodium dodecyl sarcosinate solution at 68°C for 1 hour, followed by their exposure to X-ray films (XAR-5, Kodak) overnight at -80°C. After development of the films, 6 positive signals were found from the liver cDNA library, and 5 positive signals from the placenta cDNA library. By subjecting these clones to second and third screenings, 4 positive clones were isolated from the liver cDNA library, and 3 positive clones from the placenta cDNA library. The *Eco*RI insert DNA fragments of the obtained 7 clones were separately subcloned into an *Eco*RI site of plasmid vector pUC19 in the same manner as described above in order to make restriction maps for the inserts of the 7 clones. As the results, 4 clones obtained from the liver cDNA library and 2 clones from the placenta cDNA library were found to overlap one another. In addition, it was estimated that the full length human PDI cDNA desired is covered by one of the liver-originated clones (pHDPI16) and one of the placenta-originated clones (pHDPIp4) based upon the comparison of restriction maps of these clones with that reported by Pihlajaniemi *et al*. DNA base sequences of the two clones were determined using

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M13 SEQUENCING KIT (Toyobo, Japan.), M13 Sequencing Kit (Takara Shuzo) and an automatic DNA sequencer (370A, Applied Biosystems). Comparison of the thus determined sequences with the data reported by Pihlajaniemi *et al*. confirmed that the full length human PDI cDNA which consists of 2454 base pairs is encoded by these two clones (SEQ ID NO:1).

Construction of plasmid for human PDI expression in yeast

A plasmid for use in the expression of human PDI in yeast was constructed by the following procesure, using the above two clones, pHPDI16 and pHPDIp4, which encode human PDI cDNA (Figs. 1A, 1B and 1C):

About 1 µg of pHPDI16 DNA prepared by the alkaline lysis method was digested at 37°C for 1 hour in 20 µl of the digestion solution consisting of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$, 6 mM mercaptoethanol, 0.1% gelatin, 10 units of *Eco*RI (Nippon Gene) and 10 units of *Pst*I (Nippon Gene). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then to the glass powder method to separate and purify a DNA fragment of about 490 bp which corresponds to a 5'-end *Eco*RI-*Pst*I fragment of the PDI cDNA. Separately from this, about 1 µg of pHPDIp4 DNA was digested at 37°C for 1 hour in 20 µl of the digestion solution consisting of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM $MgCl_2$, 6 mM mercaptoethanol, 0.1% gelatin, 10 units of *Pst*I (Nippon Gene) and 10 units of *Bam*HI (Nippon Gene). The resulting digest was treated in the same manner as described above to separate and purify a DNA fragment of about 1.3 kb which corresponds to a 3'-end *Pst*I-*Bam*HI fragment of the PDI

cDNA. The thus recovered two DNA fragments (about 50 ng for each) were ligated with about 20 ng of plasmid vector pUC 119 which has been digested in a linear form with *Eco*RI and *Bam*HI, by incubating these DNA samples at 16°C for 15 hours in the mixture of 25 µl of Liquid A and 5 µl of Liquid B of the DNA ligation kit (Takara Shuzo). With 10 µl of the reaction mixture obtained, a competent *E. coli* strain MV1190 cell was transformed by the calcium chloride technique. The transformed cell was cultured overnight at 37°C on an X-Gal plate (LB medium containing 1.5% agar further supplemented with 50 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 30 µg/ml of isopropyl-β-D-thiogalactopyranoside and 25 µg/ml of ampicillin) of 90 mm in diameter. White colonies grown on the plate were picked up, plasmid DNAs were prepared from the colonies by the alkaline lysis method, and the DNAs were analyzed using restriction enzymes, thereby selecting a transformant which carries a target plasmid. The thus obtained plasmid was named phPDIEB.

Using the plasmid phPDIEB, a *Nae*I cleavage site was introduced into the boundary region between the PDI signal sequence and the PDI sequence itself on the cDNA by the method of Kunkel (Kunkel, T.A., *Proc. Natl. Acad. Sci.*, USA, vol.82, p.488, 1985). *E. coli* strain JW313 competent cell was transformed with the phPDIEB DNA by calcium chloride technique. A single colony of the resultant transformant was pre-cultured overnight at 37°C in 2 x YT medium (1.6% Bacto-Tryptone, 0.5% NaCl and 1% Bacto-Yeast Extract) supplemented with 150 µg/ml of ampicillin. One ml of the pre-culture was inoculated into 50 ml of the 2 x YT medium supplemented with 150 µg/ml of ampicillin, followed by its culture at 37°C. When turbidity (OD_{600}) of the medium reached around 0.3, M13K07 phage (m.o.i.= 2) was added to the medium, and the infection was carried out by incubating the mixture at 37°C for 30 minutes without shaking. To this cell suspension was added kanamycin to a final concentration of 70 µg/ml, followed by culturing at 37°C for 20 hours with shaking. The obtained culture was subjected to centrifugation, and the supernatant recovered was mixed with 1/5 volumes of a solution containing 2.5 M NaCl and 20% polyethylene glycol #6000. After stirring, the mixture was left for 15 minutes at room temperature. The precipitate obtained by centrifugation was dissolved in 5 ml of the TE buffer (pH 8.0) consisting of 10 mM Tris-HCl and 1 mM EDTA, mixed with an equal volume of neutral phenol with stirring, and then centrifuged to recover an aqueous layer. To the layer was added an equal volume of chloroform with stirring. The mixture was further subjected to centrifugation to recover an aqueous layer. The aqueous layer was then mixed with 1/10 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. After stirring, the mixture was left for 30 minutes at -80°C, followed by centrifugation in order to recover DNA as precipitate. The DNA was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 100 µl of the TE buffer.

Using the resulting phPDIEB-originated single-stranded DNA containing dU, a desired mutation, i.e., introduction of a *Nae*I site, was carried out in the following manner:

10 pmol of a synthetic oligonucleotide (5'-CGGGGGCGCCGGCGCGC-3', Takara Shuzo) for use in the introduction of a mutation was incubated at 37°C for 15 minutes in 10 µl of the phosphorylation

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solution which consists of 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 7 mM dithiothreitol, 1 mM ATP and 10 units of T4 polynucleotide kinase (Takara Shuzo), followed by heating at 70°C for 10 minutes in order to deactivate the T4 polynucleotide kinase. Separately from this, 0.2 pmol of the above-described phPDIEB-originated single-stranded DNA and 1 µl of an annealing buffer (Site-directed mutagenesis system Mutan™-K, Takara Shuzo) were mixed with sterile water to a final volume of 10 µl. One µl of this solution was mixed with 1 µl of the phosphorylated synthetic oligonucleotide solution obtained above, and the mixture was left at 65°C for 15 minutes and then at 37°C for 15 minutes. Thereafter, a complementary chain synthesis was carried out by mixing the reaction mixture with 25 µl of a chain elongation solution (Site-directed mutagenesis system Mutan™-K, Takara Shuzo), 60 units of *E. coli* DNA ligase (Mutan™-K, Takara Shuzo) and 1 unit of T4 DNA polymerase (Mutan™-K, Takara Shuzo), and by incubating the resulting mixture at 25°C for 2 hours. The reaction was terminated by adding 3 µl of 0.2 M EDTA (pH 8.0) and heating the mixture at 65°C for 5 minutes. 3 µl of the DNA solution obtained was mixed with 30 µl of a suspension of *E. coli* strain BMH71-18mutS competent cell, and the cell suspension was left for 30 minutes in an ice bath, for 45 seconds at 42°C and then for 1 minute in an ice bath. To the cell suspension was then added 300 µl of SOC medium (2% Bacto-trypton, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$ and 20 mM glucose), and the mixture was shaken at 37°C for 1 hour. 10 µl of M13K07 phage was further added thereto, and the mixture was left for 30 minutes at 37°C. After adding 1 ml of 2 x YT medium containing 150 µg/ml of

ampicillin and 70 µg/ml of kanamycin to the mixture, the mixture was shaken at 37°C for 20 hours. The resulting culture was centrifuged to recover 20 µl of supernatant which was subsequently mixed with 80 µl of a culture of *E. coli* strain MV1190. After incubation at 37°C for 10 minutes, the resulting mixture was inoculated onto a LB plate supplemented with 150 µg/ml of ampicillin and cultured overnight at 37°C. Among colonies grown on the plate, a transformant carrying a *NaeI* site-introduced plasmid was identified by DNA-sequencing using M13 SEQUENCING KIT (Toyobo). This plasmid was named phPDINae.

2 µg of the phPDINae DNA prepared by the alkaline lysis method was digested at 37°C for 4 hours in 30 µl of the digestion solution consisting of 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 7 mM $MgCl_2$, 7 units of *NaeI* (Nippon Gene) and 10 units of *HindIII* (Takara Shuzo). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique so as to separate and purify a DNA fragment of about 1.7 kb.

A plasmid, pUC119Sig, containing a DNA fragment which encodes a human serum albumin prepro-sequence and is composed of codons often utilized in yeast was constructed in the following manner (Fig. 1A):

One µg of plasmid vector pUC119 DNA was digested at 37°C for 1 hour in 20 µl of the digestion solution which consists of 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 12 units of *EcoRI* (Nippon Gene), followed by heating at 70°C for 5 minutes in order to deactivate the enzyme. To the reaction mixture was added 38 µl of sterile water and 1 unit of bacterial alkaline phosphatase (Takara Shuzo), and the mixture was incubated at 37°C for 1 hour, followed by phenol extraction and ethanol precipitation to recover DNA. The

DNA was then incubated overnight at 16°C in 30 µl of the ligation solution which consisted of 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP and 300 units of T4 DNA ligase (Takara Shuzo), together with an equal molar amount of a *Xho*I linker containing a *Xho*I site and consisting of the following sequence:

5'-AATTCTCGAG

GAGCTCTTAA-5'.

Using 10 µl of this solution, transformation of *E. coli* JM107 competent cell was carried out by the calcium chloride method. The transformed cell was cultured overnight at 37°C on a LB plate supplemented with 50 µg/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis method from the colonies on the plate and analyzed using restriction enzymes. In this way, a plasmid DNA molecule in which the *Xho*I linker has been inserted into pUC119 *Eco*RI site was selected.

The following four types of oligonucleotides:

(1) 5'-TCGAGAATTCATGAAGTGGGTTACCTTCATCTCTTTGTTGTT-3';

(2) 5'-AACAAGAACAACAAAGAGATGAAGGTAACCCACTTCATGAATTC-3';

(3) 5'-CTTGTTCTCTTCTGCTTACTCTAGAGGTGTTTTCAGAAGGCCTG-3'; and

(4) 5'-GATCCAGGCCTTCTGAAAACACCTCTAGAGTAAGCAGAAGAG-3'

were synthesized using an automatic DNA synthesizer (380B, Applied Biosystems).

Each 5'-end of these oligonucleotides was phosphorylated by incubating about 30 pmol of each sample at 37°C for 1 hour in the solution consisting of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM ATP and 6 units of T4 polynucleotide kinase (Takara Shuzo). The oligonucleotide solutions obtained were combined (100 µl in total volume) and annealed by leaving the combined solution for 5 minutes in a water bath of 100°C, followed by cooling down to room temperature. To the solution was then added 600 units of T4 DNA ligase (Takara Shuzo), and the mixture was left overnight at 16°C to ligate these fragments. The double-stranded DNA preparation thus obtained was subjected to phenol extraction in order to remove proteins, and then to ethanol precipitation to recover the DNA.

One µg of the above *Xho*I linker-introducing vector plasmid was digested at 37°C for 1 hour in 20 µl of the digestion solution consisting of 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 10 units of *Bam*HI (Nippon Gene) and 12 units of *Xho*I (Takara Shuzo), followed by phenol extraction and ethanol precipitation to recover a DNA fragment. The fragment obtained was incubated overnight at 16°C in 30 µl of the ligation solution which consists of 66 mM Tris-HCl (pH 7.5), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP and 300 units of T4 DNA ligase (Takara Shuzo), together with an equal molar amount of the double-stranded DNA fragment obtained by ligating the four oligonucleotides. Using 10 µl of the thus prepared solution, transformation of *E. coli* JM107 competent cells was carried out by the calcium chloride method. The transformed cells were cultured overnight at 37°C on LB medium containing 50 µg/ml of ampicillin. Plasmid DNAs prepared from colonies on the plate were analyzed using restriction enzymes so as to select a transformant containing a desired recombinant plasmid. The obtained plasmid was named pUC119Sig.

A DNA was prepared from plasmid pUC119Sig by the alkaline lysis method. 2 µg of the DNA was digested at 37°C for 4 hours in the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM $MgCl_2$, 8 units of *Stu*I (Nippon Gene) and 10 units of *Hind*III (Takara Shuzo), subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 3.2 kb. The 1.7 kb DNA fragment (about 50 ng) derived from phPDINae was reacted with the 3.2 kb DNA fragment (about 50 ng) from pUC119Sig at 16°C for 30 minutes in the ligation kit solution of Takara Shuzo(Japan) (a mixture of Liquid A 30 µl + Liquid B 5 µl). Using 10 µl of its reaction mixture, transformation of *E. coli* HB101 competent cells (Takara Shuzo) was carried out by the calcium chloride method. The transformed cells were cultured overnight at 37°C on a LB plate supplemented with 50 µg/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis method from colonies grown on the plate and analyzed using restriction enzymes to select a recombinant plasmid in which the human PDI itself was linked to the downstream side of the human serum albumin prepro-sequence (Fig. 2). The obtained plasmid was named phPDILyl.

A human PDI expression plasmid was constructed in the following manner, such that the leader sequence modified type PDI can express under the control of a promoter of yeast alcohol dehydrogenase I gene:

7 µl of phPDILyl DNA prepared by the alkaline lysis method was digested at 37°C for 2 hours in 100 µl of the digestion solution which consists of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM $MgCl_2$

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and 40 units of *Eco*RI (Nippon Gene). The resulting solution was mixed with an equal volume of a phenol/chloroform mixture (a mixture of saturated phenol with an equal volume of chloroform). After stirring, the mixture was centrifuged to recover an aqueous layer. The phenol/chloroform extraction was repeated, and the aqueous layer obtained was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 2 hours at -40°C and then subjected to centrifugation. The pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50 µl of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the solution obtained was added 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt-end the *Eco*RI cleavage site. The thus prepared solution was subjected twice to phenol/chloroform extraction, and the resulting aqueous layer was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 1 hour at -40°C and then subjected to centrifugation. The pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50 µl of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the solution obtained was added 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt the *Eco*RI cleavage site. The reaction mixture was then subjected twice to phenol/chloroform extraction, and the resulting aqueous layer was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 1 hour at -40°C and then centrifuged. The pellet was

washed with 70% ethanol, dried under a reduced pressure and then dissolved in 40 µl of the solution which consists of 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 7 mM $MgCl_2$ and 10 units of *BamHI* (Nippon Gene). Thereafter, the DNA solution obtained was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique so as to separate and purify a DNA fragment of about 1.8 kb. Separately from this, 5 µl of pJDB-ADH-HSA-A DNA (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384) prepared by the alkaline lysis method was digested at 37°C for 2 hours in 100 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM $MgCl_2$ and 24 units of *XhoI* (Takara Shuzo). The reaction mixture was then subjected twice to phenol/chloroform extraction, and the aqueous layer separated was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 2 hours at -40°C and then centrifuged to recover DNA as precipitate. The DNA precipitate was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50 µl of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the solution obtained was added 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt the *XhoI* cleavage site. The reaction mixture was then subjected twice to phenol/chloroform extraction, and the aqueous layer separated was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. This mixture was left for 1 hour at -40°C before centrifugation. The DNA pellet removed was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 40 µl of the solution which consists of 10 mM

Tris-HCl (pH 8.0), 60 mM NaCl, 7 mM $MgCl_2$ and 10 units of *BamHI* (Nippon Gene). The solution obtained was incubated at 37°C for 75 minutes to digest the DNA. To the reaction mixture was then added 10 µl of 2 M Tris-HCl (pH 8.0), 110 µl of sterile water and 1 unit of alkaline phosphatase from *E. coli* strain C75 (Takara Shuzo), and the mixture was incubated at 60°C for 1 hour in order to carry out a 5'-end dephosphorylation of the restriction enzyme-formed cleavage site. To the reaction mixture was added 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture obtained was left for 1 hour at -40°C before centrifugation. The DNA pellet separated was dried under a reduced pressure and then dissolved in 20 µl of the TE buffer. Thereafter, the prepared DNA solution was subjected to 0.8% agarose gel electrophoresis and then treated by the powder glass technique so as to separate and purify a DNA fragment of about 8 kb. The thus obtained phPDILyl-originated 1.8 kb DNA fragment (about 50 ng) and pJDB-ADH-HSA-A-originated 8 kb DNA fragment (about 50 ng) were incubated at 16°C for 2.5 hours in the DNA ligation kit solution of Takara Shuzo (a mixture of Liquid A 30 µl + Liquid B 6 µl) in order to ligate the two DNA fragments. Using 10 µl of the prepared DNA solution, transformation of *E. coli* strain C600 was carried out by the calcium chloride technique. The transformed cells were cultured overnight at 37°C on a LB plate supplemented with 50 µg/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis method from colonies grown on the plate and analyzed using restriction enzymes in order to select a transformant carrying a plasmid in which the leader sequence modified type PDI sequence was linked to the downstream side of the

alcohol dehydrogenase I promoter. The constructed PDI expression plasmid was named pAHhPDILyl. As the results of the plasmid construction, the N-terminal amino acid of the mature PDI protein was changed from Asp to Gly.

A control plasmid for use in experiments of the human PDI expression was constructed in the following manner:

5 µl of pJDB-ADH-HSA-A DNA prepared by the alkaline lysis method was digested at 37°C for 2 hours in 100 µl of the digestion solution which consists of 10 mM Tris-HCl, 100 mM NaCl, 7 mM $MgCl_2$, 24 units of *Xho*I (Takara Shuzod) and 29 units of *Bam*HI (Nippon Gene). The reaction mixture obtained was subjected twice to phenol/chloroform extraction, and to the aqueous layer was added 1/10 vol of 3 M sodium acetate (pH 5.3) and 2.5 vol of ethanol. The mixture obtained was then left for 2 hours at -40°C and then centrifuged to recover DNA as pellet. The DNA pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50 µl of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the obtained solution was added a 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt the *Xho*I and *Bam*HI cleavage sites. The reaction mixture was then subjected twice to phenol/chloroform extraction, and the aqueous layer separated was mixed with 1/10 vol of 3 M sodium acetate (pH 5.3) and 2.5 vol of ethanol. The mixture was left for 1 hour at -40°C before centrifugation. The DNA pellet removed was dried under a reduced pressure and dissolved in 20 µl of the TE buffer. Thereafter, the DNA solution was subjected to 0.8% agarose gel electrophoresis and

then treated by the glass powder technique to separate and purify a DNA fragment of about 6 kb. The thus obtained DNA fragment (about 50 ng) was mixed with the mixture of Liquid A 30 µl + Liquid B 6 µl from the DNA ligation kit (Takara Shuzo), and incubated overnight at 16°C so as to cyclize it by self-ligation. Using 10 µl of the prepared DNA solution, transformation of *E. coli* 101 competent cells (Takara Shuzo) was carried out by the calcium chloride technique. The transformed cells were cultured overnight at 37°C on a LB plate supplemented with 50 µl/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis technique from the colonies grown on the plate, and analyzed using restriction enzymes in order to select a desired control plasmid. The constructed plasmid was named pAH.

Expression of human PDI in yeast

Using the human PDI expression plasmid pAHhPDILyl constructed above, an expression of human PDI in yeast was carried out in the following manner:

A single colony of yeast strain AH22 obtained by culturing it on a YPD plate (2% Bacto-pepton, 1% yeast extract, 2% glucose and 1.5% agar) was inoculated into 5 ml of a YPD medium (2% Bacto-pepton, 1% yeast extract and 2% glucose) and cultured at 30°C for 24 hours with shaking. This pre-culture (0.9 ml) was then inoculated into 45 ml of the YPD medium and cultured at 30°C with shaking. When turbidity at OD_{600} reached about 0.5, the main culture was subjected to a low speed centrifugation to recover yeast cells as precipitate. The cells removed were suspended in 3 ml of 0.2 M LiSCN, and the cell suspension (1 ml) was centrifuged to recover the

cells. To the cells were subsequently added 46 µl of 50% PEG #4000, 10 µl of LiSCN and 10 µl of a pAHhPDILyl DNA solution (27 µg as DNA) prepared by the alkaline lysis method. After mixing them by pipetting, the mixture was left overnight at 30°C, followed by its suspension in 1 ml of sterile water. The suspension was then centrifuged to recover cells as pellet. The pellet was resuspended in 100 µl of sterile water and cultured at 30°C after the inoculation of its suspension onto a SD(-Leu) plate (SD(-Leu) medium (0.67% Bacto-nitrogen base, 2% glucose, 20 mg/l of adenine, 20 mg/l of uracil, 20 mg/l of tryptophan, 20 mg/l of histidine, 20 mg/l of arginine, 20 mg/l of methionine, 30 mg/l of tyrosine, 30 mg/l of isoleucine, 30 mg/l of lysine, 50 mg/l of phenylalanine, 100 mg/l of aspartic acid, 100 mg/l of glutamic acid, 150 mg/l of valine, 200 mg/l of threonine and 375 mg/l of serine (amino acids from Wako Pure Chemical Industries, Japan)) + 1.5% agar). A transformant from the 5-days culture was inoculated into 5 ml of the SD (-Leu) medium and cultured at 30°C for 2 days with shaking. 100 µl of the obtained pre-culture was then inoculated into 5 ml of the YPD medium and cultured at 30°C for 24 hours with shaking. 1.5 ml of the resulting main culture was centrifuged to recover 500 µl of supernatant which was subsequently mixed with the equal volume of ethanol and then left for 1 hour in an ice bath. The mixture was centrifuged so as to recover products secreted out of the yeast cells as a pellet which was then dried under a reduced pressure. The pellet was dissolved in 10 µl of a sample buffer for SDS-PAGE (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.01% Bromophenol Blue). After boiling for 5 minutes, the treated sample

was subjected to electrophoresis on SDS/PAGE Plate 10/20 (Daiichi Kagaku Yakuhin, Japan). The resulting gel was stained with a staining solution (0.15% Coomassie Brilliant Blue, 10% acetic acid and 40% methanol) and then soaked in a decoloring solution (10% acetic acid and 40% methanol) to visualize an expressed product. In this instance, a control sample obtained by the same procedure, except that pAHhPDILyl was replaced by the aforementioned control plasmid pAH, was run at the same time during the electrophoresis. As standard molecular weight markers, phosphorylase b (molecular weight, 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,000) were used (Fig. 3). As the results, an expression product having a molecular weight of about 55 K was found. Since this molecular weight coincided with that of the mature PDI protein, it was assumed that a desired human PDI was expressed and secreted. Next, a large-scale culture was carried out in the following manner in order to examine chemical properties of the expressed and secreted protein:

A single colony of the pAHhPDILyl-carrying yeast strain AH22 was inoculated into 80 ml of the SD (-Leu) medium and cultured at 30°C for 2 days with shaking. The obtained pre-culture was then inoculated into 4 liters of a YPD-phosphate medium (YPD medium, 6 g/l of Na_2HPO_4, and 3 g/l of KH_2PO_4, pH 7.0) and cultured at 30°C for 24 hours with shaking. The resulting main culture was centrifuged to removed the supernatant which was used for the purification of the secreted expression product.

Isolation of recombinant human PDI from the culture and its characterization

The culture (4 liters) obtained by culturing the recombinant yeast was concentrated to 1/40 (final volume, 100 ml) using a Millipore-Millitan ultrafiltration apparatus (nominal molecular weight, 30,000 cut-off), and then subjected to TSK-gel Phenyl-5PW hydrophobic column chromatography so as to isolate human PDI. The elution was carried out in 10 mM borate-10 mM KCl buffer (pH 8.0) containing 0.05% NaN_3, with a linear gradient from 0.85 M to 0 M of ammonium sulfate over 125 minutes. The flow rate was 2 ml/min. The result is shown in Fig. 4. In Fig. 5 the result of SDS-electrophoresis of the isolated human PDI is illustrated. As shown in the figures, the human PDI was purified almost homogeneously by the hydrophobic column chromatography without a loss of its activity. Any UV-absorbing substance in the YPD medium could be removed markedly efficiently by the chromatography.

PDI assay

PDI assay was carried out by measuring its effect to enhance the refolding of scrambled ribonuclease A (RNase A) which has been prepared by reduction, denaturation and re-oxidation steps. Refolding degree of the scrambled RNase A was determined by measuring a degree of the restoration of its enzyme activity. The following describes the assay procedure illustratively:

Preparation of scrambled RNase A:

120 mg of RNase A was dissolved in 3 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 6 M guanidine hydrochloride and 0.1% M dithiothreitol, and then reduced under nitrogen atmosphere at room temperature for 15 hours. The reduced product was applied to a Sephadex G-25 column (15 mmø x 30 cm) equilibrated with 0.01 N HCl, thereby removing the reducing agent. To the desalting product was added guanidine hydrochloride to a final concentration of 6 M. After adjusting its pH value to 9.0 with Tris, the mixture was subjected to an exchange reaction of a S-S bond(s) in the dark at 4°C for 14 days. The thus prepared sample was stored at -80°C for use as the scrambled RNase A.

PDI assay:

10 µl of 1 M dithiothreitol is added to 20 ml of 55 mM phosphate buffer (pH 7.5) in which any dissolving air was replaced with nitrogen gas. 10 µl of this solution is added to 420 µl of 55 mM phosphate buffer (pH 7.5) mixed with 20 µl of an enzyme sample, and the mixture is left for 5.5 minutes at 30°C. To this solution is added 50 µl of the scrambled RNase solution prepared above, followed by the enzymatic reaction at 30°C for 15.5 minutes. Separately from this, 1.945 ml of degassed 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 25 mM KCl and 50 µl of a yeast RNA solution (dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA; adjusted to such a concentration that absorbance at 280 nm becomes 80) is placed into a quartz cell (1cm x 1cm). With stirring, a temperature of the resulting mixture is maintained at 45°C. In this instance, absorbance at 260 nm should not be changed by the treatment. Thereafter, 5 µl of the dithiothreitol-treated scrambled RNase A solution is added to the buffer in the quartz cell and, with stirring, a change in the absorbance of the reaction

mixture at 260 nm are measured over 2 minutes at 0.2 minute intervals. The PDI activity is calculated from an initial velocity of the changing rate of absorbance at 260nm.

Transformation of yeast strain HIS23 with human PDI expression plasmid pAHhPDILyl

Using the aforementioned human PDI expression plasmid pAHhPDILyl, transformation of the HSA-producing yeast strain HIS23 (Japanese Patent Application No. 2-57885 filed by the present applicant, Bikoken-Kin-Ki No. 11351 (FERM P-1138)) was carried out in the following manner:

A single colony of the HSA-expressing yeast strain HIS23 obtained by culturing it on a YPD plate (2% Bacto-trypton, 1% Bacto-yeast extracts, 2% glucose and 1.5% agar) was inoculated into 5 ml of a YPD medium (2% Bacto-trypton, 1% yeast extract and 2% glucose) and cultured at 30°C for 24 hours with shaking. One ml of the obtained pre-culture was inoculated into 50 ml of the YPD medium and cultured at 30°C with shaking. When turbidity at OD_{600} reached about 0.5, the main culture was subjected to a low speed centrifugation to recover the yeast cells as pellet. To the pellet were added 46 µl of 50% polyethylene glycol #4000, 10 µl of LiSCN and 10 µl of the human PDI expression plasmid pAHhPDILyl DNA solution (about 20 µg as DNA) prepared by the alkaline lysis method (Birnboim, H.C. and Doly, J., *Nucleic Acids Res.*, vol.7, p.1513, 1979). After mixing them by pipetting, the mixture was left overnight at 30°C. The resulting mixture was suspended in 1 ml of sterile water and centrifuged to recover cells as pellet. The pellet was suspended in 100 µl of

sterile water and cultured at 30°C after inoculating the cell suspension onto a SD (-His,-Leu) plate (0.67% Bacto-nitrogen base, 2% glucose, 20 mg/l of adenine, 20 mg/l of uracil, 20 mg/l of tryptophan, 20 mg/l of arginine, 20 mg/l of methionine, 30 mg/l of tyrosine, 30 mg/l of isoleucine, 30 mg/l of lysine, 50 mg/l of phenylalanine, 100 mg/l of aspartic acid, 100 mg/l of glutamic acid, 150 mg/l of valine, 200 mg/l of threonine and 375 mg/l of serine (amino acids from Wako Pure Chemical Industries)) + 1.5% agar). A transformant was obtained as a colony grown on the plate on the day 5 after the culture.

Expression of PDI in the obtained transformant (pAHhPDILyl/HIS23) was examined in the following manner:

In this instance, a transformant (pAH/HIS23) obtained using the plasmid pAH which has been prepared by removing the PDI cDNA moiety from pAHhPDILyl was used as a control. The single colony grown on the plate was inoculated into 5 ml of the SD (-His, -Leu) medium and cultured at 30°C for 2 days with shaking. 100 µl of the pre-culture was then inoculated into 5 ml of the YPD medium and cultured at 30°C for 24 hours with shaking. 1.5 ml of the resulting main culture was centrifuged to recover 500 µl of supernatant which was subsequently mixed with the equal volume of ethanol and then left for 1 hour in an ice bath. The mixture was centrifuged to recover products secreted out of the yeast cells as a precipitate which was then dried under a reduced pressure using an evaporator. The precipitate was dissolved in 10 µl of a sample buffer for SDS-PAGE (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 0.005% Bromophenol Blue and 20% glycerol). After boiling for 5 minutes, the sample was

subjected to electrophoresis on SDS-PAGE Plate 4/20-1010 (ex Daiichi Kagaku Yakuhin). The resulting gel was stained with a staining solution (0.15% Coomassie Brilliant Blue, 10% acetic acid and 40% methanol) and then soaked in a decoloring solution (10% acetic acid and 40% methanol) to visualize an expressed product. In this instance, phosphorylase b (molecular weight, 94,000 daltons), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,000) were used as standard molecular weight markers (Fig. 6). As the results, the secretion of an expressed PDI having a molecular weight of about 55,000 daltons was detected in the yeast strain HIS23 transformed with pAHhPDILy1.

Effect of human PDI on the expression and secretion of HSA

Using the above-described co-expression system of HSA and PDI in a yeast cell, effects of human PDI on the expression and secretion of HSA were examined in the following manner:

Five single colonies were isolated from strain pAH/HIS23 which has been obtained by transforming the yeast strain HIS23 with the control plasmid pAH, and other 5 single colonies were isolated from strain pAHhPDILy1/HIS23 obtained by transforming the HIS23 with the human PDI expression plasmid pAHhPDILy1. Each of the thus isolated single colonies was inoculated into 5 ml of the SD (-His, -Leu) medium and cultured at 30°C for 24 hours. Each of the pre-cultures (100 µl) was inoculated into 5 ml of the YPD medium and cultured at 30°C for 24 hours with shaking. From the main cultures obtained, samples for SDS-PAGE were prepared in accordance with the

aforementioned procedure. Results of the SDS-PAGE are shown in Fig. 7. Using the gels subjected to the SDS-PAGE, the amount of secreted HSA from each strain was determined using a densitometer (IMAGE ANALYSIS SYSTEM, ex TEFCO, Japan) in order to examine effects of the co-expression of PDI on the secreted amount of expressed HSA (Fig. 8). As shown in the figure, the strains pAH/HIS23 and pAHhPDILy1 secreted HSA in average amounts of 0.93 mg/l and 1.50 mg/l, respectively. In other words, secretion of HSA was increased by about 60% in average due to the co-expression of PDI in the yeast strain HIS23.

[Advantages of the Invention]

According to the present invention, a process for the large-scale preparation of human PDI has been fast established by utilizing a fusion gene composed of a DNA fragment coding for human serum albumin pre-pro sequence and a human protein disulfide isomerase gene. Accordingly, this process can be used as a means to enhance the activation of a protein in which the formation of a high-order structure thereof is incomplete because of the mistaken bond formation of a S-S bond(s), and thus as a means to prepare a large amount of the protein economically. A main advantage of the invention is the activation of an inactivated protein produced by means of genetic engineering techniques, and it is possible to improve a production efficiency of an other useful polypeptide in a host cell by coupling both the expressions of the enzyme and the useful polypeptide. In addition, the PDI can be used as a reagent for research.

SEQUENCE LISTING

SEQ ID NO: 1

SEQUENCE LENGTH: 2454

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

IMMEDIATE SOURCE: human liver and placenta λgt11 cDNA libraries

(Clontech)

SEQUENCE DESCRIPTION:

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                                                    -15

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Pro Glu Glu Glu Asp His Val Leu Val Leu Arg Lys Ser Asn Phe Ala
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CAG GCG CTG GCG GCC CAC AAG TAC CTG CTG GTG GAG TTC TAT GCC CCT     201
Glu Ala Leu Ala Ala His Lys Tyr Leu Leu Val Glu Phe Tyr Ala Pro
 20                  25                  30

TGG TGT GGC CAC TGC AAG GCT CTG GCC CCT GAG TAT GCC AAA GCC GCT     249
Trp Cys Gly His Cys Lys Ala Leu Ala Pro Glu Tyr Ala Lys Ala Ala
35                  40                  45                  50

GGG AAG CTG AAG GCA GAA GGT TCC GAG ATC AGG TTG GCC AAG GTG GAC     297
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Gly Lys Leu Lys Ala Glu Gly Ser Glu Ile Arg Leu Ala Lys Val Asp
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            70                  75                  80

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TAT ACA GCT GGC AGA GAG GCT GAT GAC ATC GTG AAC TGG CTG AAG AAG     441
Tyr Thr Ala Gly Arg Glu Ala Asp Asp Ile Val Asn Trp Leu Lys Lys
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CGC ACG GGC CCG GCT GCC ACC ACC CTG CCT GAC GGC GCA GCT GCA GAG     489
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GTG GAG TCG GAC TCT GCC AAG CAG TTT TTG CAG GCA GCA GAG GCC ATC     585
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ACA GCC CCG AAG ATT TTT GGA GGT GAA ATC AAG ACT CAC ATC CTG CTG   825
Thr Ala Pro Lys Ile Phe Gly Gly Glu Ile Lys Thr His Ile Leu Leu
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TTC TTG CCC AAG AGT GTG TCT GAC TAT GAC GGC AAA CTG AGC AAC TTC   873
Phe Leu Pro Lys Ser Val Ser Asp Tyr Asp Gly Lys Leu Ser Asn Phe
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ACA GAG TTC TGC CAC CGC TTC CTG GAG GGC AAA ATC AAG CCC CAC CTG  1113
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GTC TTT GTG GAG TTC TAT GCC CCA TGG TGT GGT CAC TGC AAA CAG TTG  1257
Val Phe Val Glu Phe Tyr Ala Pro Trp Cys Gly His Cys Lys Gln Leu
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Lys Phe Leu Glu Ser Gly Gly Gln Asp Gly Ala Gly Asp Asp Asp Asp
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485 490

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CCCGCGGGAG GTTTCTCCTC TTTTTGAAAA TTCCGTCTGT GGGATTTTTA GACATTTTTC 1955

GACATCAGGG TATTTGTTCC ACCTTGGCCA GGCCTCCTCG GAGAAGCTTG TCCCCCGTGT 2015

GGGAGGGACG GAGCCGGACT GGACATGGTC ACTCAGTACC GCCTGCAGTG TCGCCATGAC 2075

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GGAGGTGGCC AAGGATGCTG GAGCTGAATC AGACGCTGAC AGTTCTTCAG GCATTTCTAT 2375

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AAAAAAAAAA CCCGAATTC 2454

SEQ ID NO: 2

SEQUENCE LENGTH: 1545

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid (semi-synthetic DNA)

SEQUENCE DESCRIPTION:

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-20 -15

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Phe Leu Phe Ser Ser Ala Tyr Ser Arg Gly Val Phe Arg Arg Gly Ala

-10 -5 1

CCC GAG GAG GAG GAC CAC GTC CTG GTG CTG CGG AAA AGC AAC TTC GCG 126

Pro Glu Glu Glu Asp His Val Leu Val Leu Arg Lys Ser Asn Phe Ala

5 10 15

GAG GCG CTG GCG GCC CAC AAG TAC CTG CTG GTG GAG TTC TAT GCC CCT 174

Glu Ala Leu Ala Ala His Lys Tyr Leu Leu Val Glu Phe Tyr Ala Pro

20 25 30

TGG TGT GGC CAC TGC AAG GCT CTG GCC CCT GAG TAT GCC AAA GCC GCT 222

Trp Cys Gly His Cys Lys Ala Leu Ala Pro Glu Tyr Ala Lys Ala Ala

35 40 45 50

GGG AAG CTG AAG GCA GAA GGT TCC GAG ATC AGG TTG GCC AAG GTG GAC 270

Gly Lys Leu Lys Ala Glu Gly Ser Glu Ile Arg Leu Ala Lys Val Asp

55 60 65

GCC ACG GAG GAG TCT GAC CTG GCC CAG CAG TAC GGC GTG CGC GGC TAT 318

Ala Thr Glu Glu Ser Asp Leu Ala Gln Gln Tyr Gly Val Arg Gly Tyr

70 75 80

CCC ACC ATC AAG TTC TTC AGG AAT GGA GAC ACG GCT TCC CCC AAG GAA 366

Pro Thr Ile Lys Phe Phe Arg Asn Gly Asp Thr Ala Ser Pro Lys Glu

85 90 95

TAT ACA GCT GGC AGA GAG GCT GAT GAC ATC GTG AAC TGG CTG AAG AAG 414

Tyr Thr Ala Gly Arg Glu Ala Asp Asp Ile Val Asn Trp Leu Lys Lys

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GTG GAG TCG GAC TCT GCC AAG CAG TTT TTG CAG GCA GCA GAG GCC ATC   558
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        165                 170                 175
TAC CAG CTC GAC AAA GAT GGG GTT GTC CTC TTT AAG AAG TTT GAT GAA   654
Tyr Gln Leu Asp Lys Asp Gly Val Val Leu Phe Lys Lys Phe Asp Glu
    180                 185                 190
GGC CGG AAC AAC TTT GAA GGG GAG GTC ACC AAG GAG AAC CTG CTG GAC   702
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195                 200                 205                 210
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                215                 220                 225
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Phe Leu Pro Lys Ser Val Ser Asp Try Asp Gly Lys Leu Ser Asn Phe
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275                 280                 285                 290
AAG AAG GAA GAG TGC CCG GCC GTG CGC CTC ATC ACC CTG GAG GAG GAG   990
Lys Lys Glu Glu Cys Pro Ala Val Arg Leu Ile Thr Leu Glu Glu Glu
            295                 300                 305
ATG ACC AAG TAC AAG CCC GAA TCG GAG GAG CTG ACG GCA GAG AGG ATC  1038
Met Thr Lys Tyr Lys Pro Glu Ser Glu Glu Leu Thr Ala Glu Arg Ile
        310                 315                 320
ACA GAG TTC TGC CAC CGC TTC CTG GAG GGC AAA ATC AAG CCC CAC CTG  1086
Thr Glu Phe Cys His Arg Phe Leu Glu Gly Lys Ile Lys Pro His Leu
    325                 330                 335
ATG AGC CAG GAG CTG CCG GAG GAC TGG GAC AAG CAG CCT GTC AAG GTG  1134
Met Ser Gln Glu Leu Pro Glu Asp Trp Asp Lys Gln Pro Val Lys Val
340                 345                 350
CTT GTT GGG AAG AAC TTT GAA GAC GTG GCT TTT GAT GAG AAA AAA AAC  1182
Leu Val Gly Lys Asn Phe Glu Asp Val Ala Phe Asp Glu Lys Lys Asn
355                 360                 365                 370
GTC TTT GTG GAG TTC TAT GCC CCA TGG TGT GGT CAC TGC AAA CAG TTG  1230
Val Phe Val Glu Phe Tyr Ala Pro Trp Cys Gly His Cys Lys Gln Leu
            375                 380                 385
GCT CCC ATT TGG GAT AAA CTG GGA GAG ACG TAC AAG GAC CAT GAG AAC  1278
Ala Pro Ile Trp Asp Lys Leu Gly Glu Thr Tyr Lys Asp His Glu Asn
        390                 395                 400
ATC GTC ATC GCC AAG ATG GAC TCG ACT GCC AAC GAG GTG GAG GCC GTC  1326
Ile Val Ile Ala Lys Met Asp Ser Thr Ala Asn Glu Val Glu Ala Val
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rg Thr Val Ile Asp Tyr Asn Gly Glu Arg Thr Leu Asp Gly Phe Lys
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AAA TTC CTG GAG AGC GGT GGC CAG GAT GGG GCA GGG GAT GAT GAC GAT   1470
Lys Phe Leu Glu Ser Gly Gly Gln Asp Gly Ala Gly Asp Asp Asp Asp
                455                 460                 465
CTC GAG GAC CTG GAA GAA GCA GAG GAG CCA GAC ATG GAG GAA GAC GAT   1518
Leu Glu Asp Leu Glu Glu Ala Glu Glu Pro Asp Met Glu Glu Asp Asp
            470                 475                 480
GAT CAG AAA GCT GTG AAA GAT GAA CTG                              1545
Asp Gln Lys Ala Val Lys Asp Glu Leu
        485                 490
```

SEQ ID NO: 3

SEQUENCE LENGTH: 491

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULAR TYPE: protein

SEQUENCE DESCRIPTION:

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Gly Ala Pro Glu Glu Glu Asp His Val Leu Val Leu Arg Lys Ser Asn
 1               5                  10                  15
Phe Ala Glu Ala Leu Ala Ala His Lys Tyr Leu Leu Val Glu Phe Tyr
            20                  25                  30
```

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Ala Pro Trp Cys Gly His Cys Lys Ala Leu Ala Pro Glu Tyr Ala Lys
        35                  40                  45
Ala Ala Gly Lys Leu Lys Ala Glu Gly Ser Glu Ile Arg Leu Ala Lys
    50                  55                  60
Val Asp Ala Thr Glu Glu Ser Asp Leu Ala Gln Gln Tyr Gly Val Arg
65                  70                  75                  80
Gly Tyr Pro Thr Ile Lys Phe Phe Arg Asn Gly Asp Thr Ala Ser Pro
                85                  90                  95
Lys Glu Tyr Thr Ala Gly Arg Glu Ala Asp Asp Ile Val Asn Trp Leu
            100                 105                 110
Lys Lys Arg Thr Gly Pro Ala Ala Thr Thr Leu Pro Asp Gly Ala Ala
        115                 120                 125
Ala Glu Ser Leu Val Glu Ser Ser Glu Val Ala Val Ile Gly Phe Phe
    130                 135                 140
Lys Asp Val Glu Ser Asp Ser Ala Lys Gln Phe Leu Gln Ala Ala Glu
145                 150                 155                 160
Ala Ile Asp Asp Ile Pro Phe Gly Ile Thr Ser Asn Ser Asp Val Phe
                165                 170                 175
Ser Lys Tyr Gln Leu Asp Lys Asp Gly Val Val Leu Phe Lys Lys Phe
            180                 185                 190
Asp Glu Gly Arg Asn Asn Phe Glu Gly Glu Val Thr Lys Glu Asn Leu
        195                 200                 205
Leu Asp Phe Ile Lys His Asn Gln Leu Pro Leu Val Ile Glu Phe Thr
    210                 215                 220
Glu Gln Thr Ala Pro Lys Ile Phe Gly Gly Glu Ile Lys Thr His Ile
225                 230                 235                 240
Leu Leu Phe Leu Pro Lys Ser Val Ser Asp Tyr Asp Gly Lys Leu Ser
```

```
                245                 250                 255
Asn Phe Lys Thr Ala Ala Glu Ser Phe Lys Gly Lys Ile Leu Phe Ile
            260                 265                 270
Phe Ile Asp Ser Asp His Thr Asp Asn Gln Arg Ile Leu Glu Phe Phe
        275                 280                 285
Gly Leu Lys Lys Glu Glu Cys Pro Ala Val Arg Leu Ile Thr Leu Glu
    290                 295                 300
Glu Glu Met Thr Lys Tyr Lys Pro Glu Ser Glu Glu Leu Thr Ala Glu
305                 310                 315                 320
Arg Ile Thr Glu Phe Cys His Arg Phe Leu Glu Gly Lys Ile Lys Pro
                325                 330                 335
His Leu Met Ser Gln Glu Leu Pro Glu Asp Trp Asp Lys Gln Pro Val
            340                 345                 350
Lys Val Leu Val Gly Lys Asn Phe Glu Asp Val Ala Phe Asp Glu Lys
        355                 360                 365
Lys Asn Val Phe Val Glu Phe Tyr Ala Pro Trp Cys Gly His Cys Lys
    370                 375                 380
Gln Leu Ala Pro Ile Trp Asp Lys Leu Gly Glu Thr Tyr Lys Asp His
385                 390                 395                 400
Glu Asn Ile Val Ile Ala Lys Met Asp Ser Thr Ala Asn Glu Val Glu
                405                 410                 415
Ala Val Lys Val His Ser Phe Pro Thr Leu Lys Phe Phe Pro Ala Ser
            420                 425                 430
Ala Asp Arg Thr Val Ile Asp Tyr Asn Gly Glu Arg Thr Leu Asp Gly
        435                 440                 445
Phe Lys Lys Phe Leu Glu Ser Gly Gly Gln Asp Gly Ala Gly Asp Asp
    450                 455                 460
```



```
Asp Asp Leu Glu Asp Leu Glu Glu Ala Glu Glu Pro Asp Met Glu Glu
465                 470                 475                 480
Asp Asp Asp Gln Lys Ala Val Lys Asp Glu Leu
                485                 490
```

Brief Description of the Drawings)

Fig. 1A illustrates a construction of the expression plasmid AHhPDILy1.

Fig. 1B illustrates a construction of the expression plasmid AHhPDILy1.

Fig. 1C illustrates a construction of the expression plasmid pAHhPDILy1.

Fig. 2 shows a boundary of the HSA prepro-sequence and the PDI gene on a human expression plasmid.

Fig. 3 is a photograph showing the result of SDS-polyacrylamide gel electrophoresis of an expressed and secreted crude recombinant human PDI, wherein lane 1 is a molecular weight marker, lane 2 is pAH/AH22 (control) and lane 3 is pAHhPDILy1/AH22.

Fig. 4 illustrates the separation of a recombinant human PDI by hydrophobic column chromatography.

Fig. 5 shows the result of SDS-polyacrylamide gel electrophoresis of a purified recombinant human PDI, wherein the numbers at the bottom correspond to the fraction numbers of the hydrophobic column chromatography shown in Fig. 4, and M is a molecular weight marker.

Fig. 6 is a photograph of SDS electrophoresis showing the expression of human PDI in a yeast strain HIS23.

Fig. 7 is a photograph of SDS-polyacrylamide gel electrophoresis showing the secretion of HSA by co-expression of human PDI and HSA in the yeast strain HIS23.

Fig. 8 shows the result of densitometric determination of the amount of secreted HSA using the SDS-polyacrylamide gel electrophoresis gel in Fig. 7.

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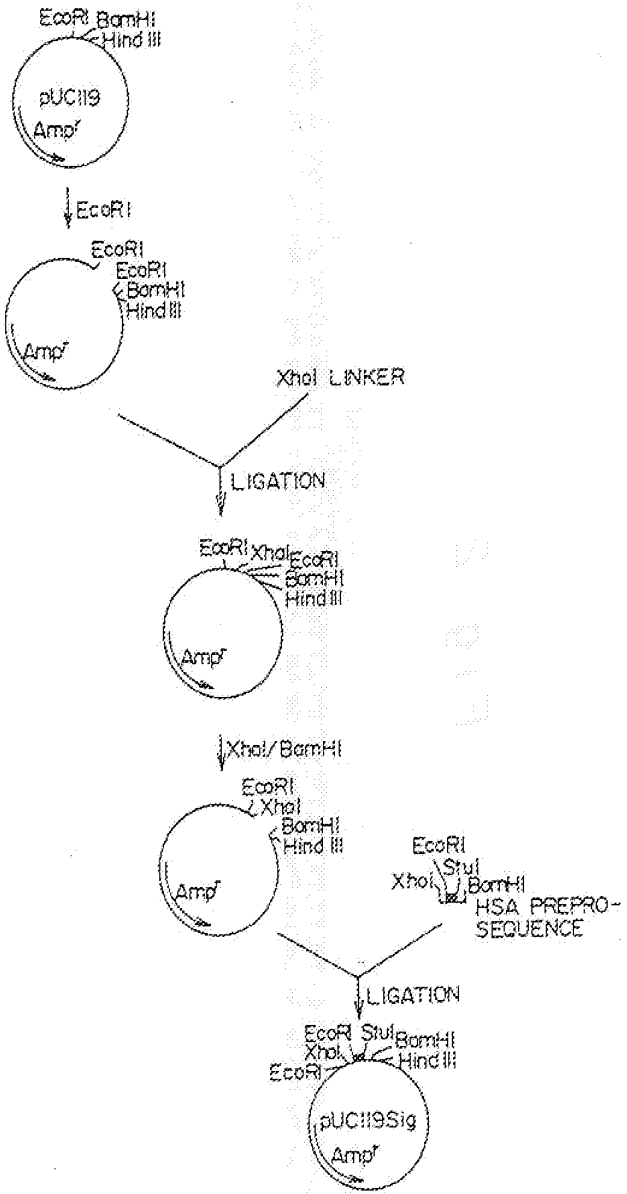
[Documents Name] Drawings

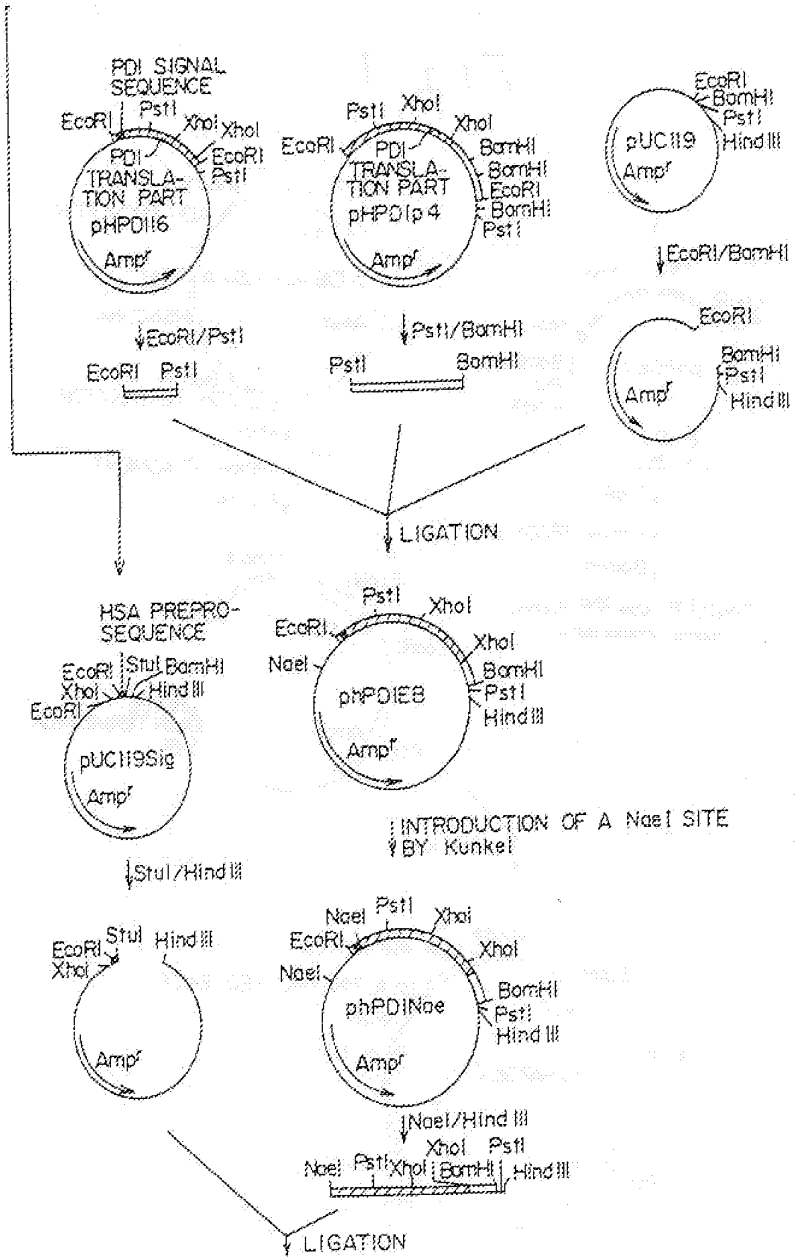
[Fig. 1]

Fig. 1 A



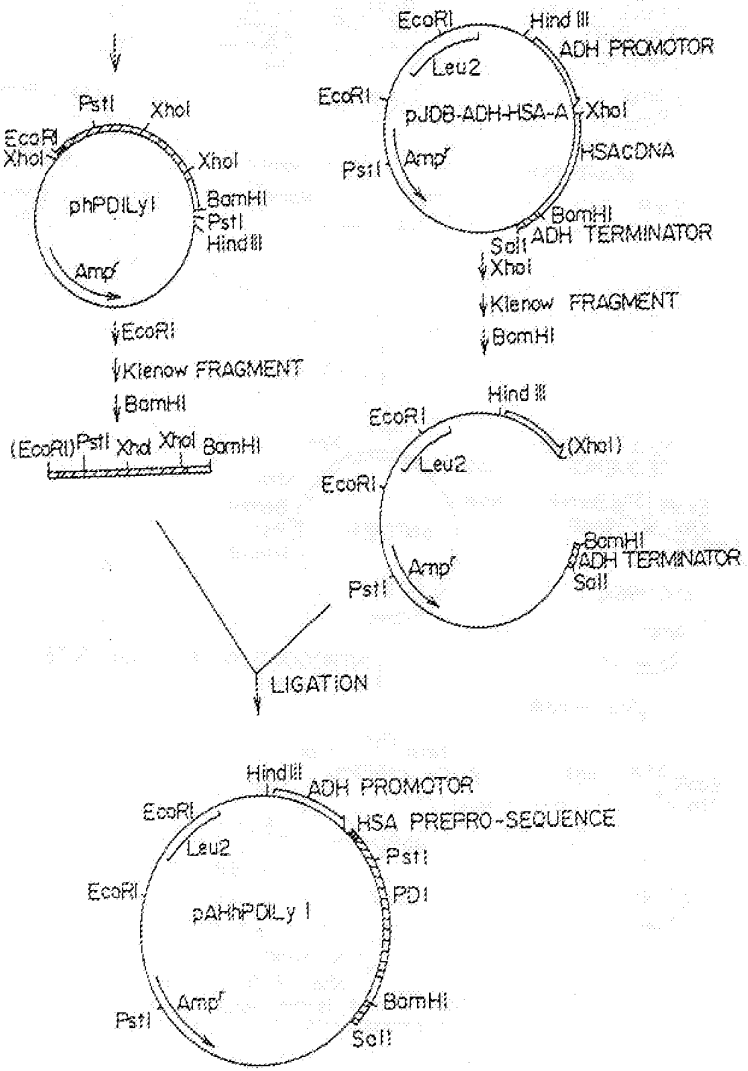
[Fig. 2]

Fig. 1 B



[Fig. 3]

Fig. 1c



[Fig. 4]

Fig. 2

HSA PREPRO-SEQUENCE

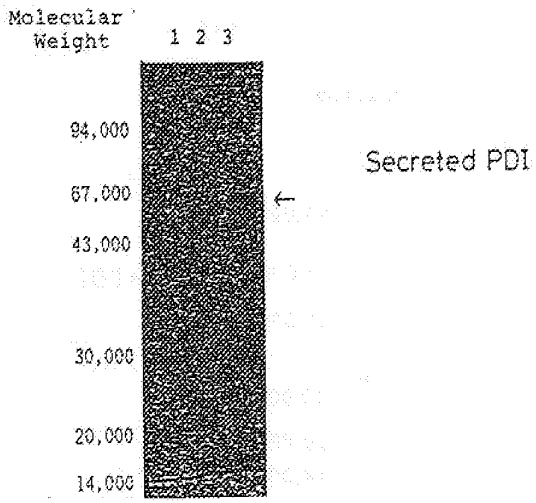
PDI

...ATG AAG TGG GTT ACC TTC ATC TCT TTG TTC TTC TCT TCT GCT TAC TCT AGA GGT GTT TTC AGA AGG GGC GCC CCC GAG GAG GAG GAC CAC...
...Met Lys Trp Val Thr Phe Ile Ser Leu Leu Phe Leu Phe Ser Ser Ala Tyr Ser Arg Gly Val Phe Arg Arg Gly Ala Pro Glu Glu Glu Asp His...

[Fig. 5]

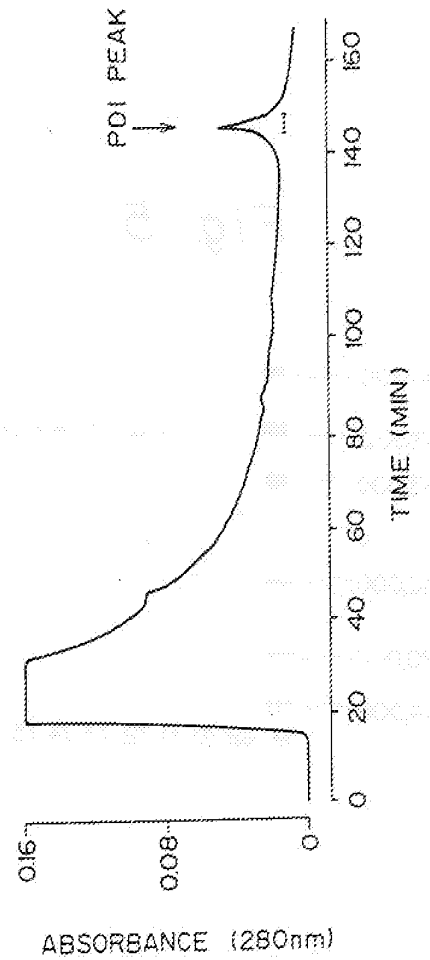
Fig. 3

Photo as substitute for drawing



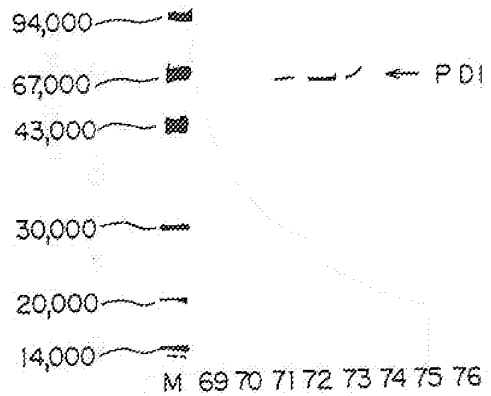
[Fig. 6]

Fig. 4



[Fig. 7]

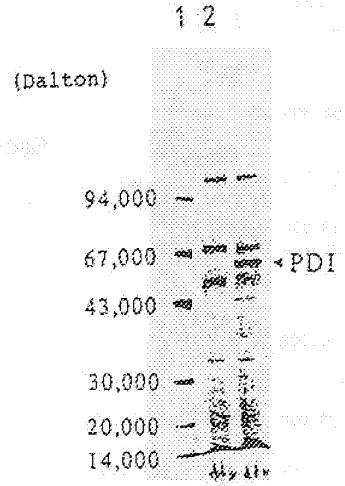
Fig. 5



[Fig. 8]

Fig. 6

Photo as substitute for drawing



1: pAH/HIS23
2: pAHhPDILyi/HIS23

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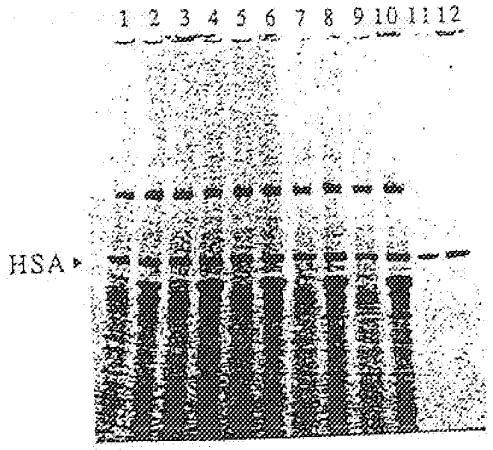
[Fig. 9]

Fig. 7

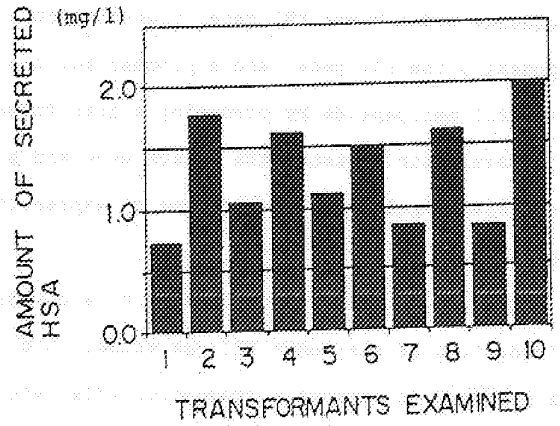
Photo as substitute for drawing



1, 3, 5, 7, 9: pAH/HIS23
2, 4, 6, 8, 10: pAHhPDILy1/HIS23
11: HSA STANDARD 0.25μg
12: HSA STANDARD 0.5μg

[Fig. 10]

Fig. 8



1, 3, 5, 7, 9: pAH/HIS23
2, 4, 6, 8, 10: pAHhPDILy1/HIS23

H 24.07.92

ABSTRACT OF THE DISCLOSURE

Abstract]

[Objects] It is an object of the present invention to provide an xpression of a protein disulfide isomerase (PDI) gene. Another bject of the invention provides a co-expression of said gene and a oreign gene coding for a useful polypeptide.

[Constitution] The present invention is characterized by a process for the preparation of PDI which comprises inserting a novel usion gene composed of a DNA fragment coding for human serum lbumin prepro sequence and a human PDI gene, transforming a host ell and then expressing the PDI gene; and a process for the reparation of a useful polypeptide by preparing a transformant ontaining, in a co-expressible state, the fusion gene and a foreign ene coding for the useful polypeptide, and then co-expressing their enes in the transformant.

[Advantages] According to the present invention, a process for he large-scale production of the human PDI was established, and ccordingly it is possible to improve a production effeciency of the seful polypeptide by the above mentioned co-expression.

Figures to be chosen] None

Information of Applicant's History

Identification Number (390022998)

1. Date of Change 16th November, 1990.

(Reason for the Change) New Registration

Address 1-1, Hitotsubashi 1-chome, Chiyoda-ku, Tokyo.

Name TONEN CORPORATION